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ORIGINAL ARTICLE

Direct access to side chain N,N'-diaminoalkylated derivatives of basic amino acids suitable for solid-phase peptide synthesis

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Abstract A simple and efficient one-pot procedure that enables rapid access to orthogonally protected N,N'-diaminoalkylated basic amino acid building blocks fully compatible with standard Boc and Fmoc solid-phase peptide synthesis is reported. Described synthetic approach includes double reductive alkylation of N^{α}-protected diamino acids with *N*-protected amino aldehydes in the presence of sodium cyanoborohydride. This approach allows preparation of symmetrical, as well as unsymmetrical, basic amino acid derivatives with branched side-chains that can be further modified, enhancing their synthetic utility. The suitability of the synthesized branched basic amino acid building blocks for use in standard solid-phase peptide synthesis has been demonstrated by synthesis of an indolicidin analogue in which the lysine residue was substituted with the synthetic derivative N^{α}-(9*H*-fluorenyl-9-methoxycarbonyl)-N^{β},$N^{\beta\prime}$-bis[2-(*tert*-butoxycarbonylamino)ethyl]-L-2,3-diaminopropionic acid. This substitution resulted in an analogue with more ordered secondary structure in 2,2,2-trifluoroethanol and enhanced antibacterial activity without altering hemolytic activity.

Keywords N-alkylation · Reductive amination · One-pot procedure · Branched basic amino acids · Solid-phase peptide synthesis · Indolicidin

Abbreviations

AcOH	Acetic acid
ACN	Acetonitrile
Aloc	Allyloxycarbonyl
Boc	*Tert*-butoxycarbonyl
CAMP	Cationic antimicrobial peptide
Cbz	Benzyloxycarbonyl
CD	Circular dichroism
CPP	Cell-penetrating peptide
DCM	Dichloromethane
EDT	1,2-Ethanedithiol
EtOAc	Ethyl acetate
Fmoc	9-Fluorenylmethoxycarbonyl
FA	Formic acid
HPLC	High-performance liquid chromatography
LPS	Lypopolysaccharide
MALDI-TOF MS	Matrix-assisted laser desorption ionization time of flight mass spectrometry
MAP	Multiantigen peptide system
MBHA	4-Methylbenzhydrylamine
MeOH	Methanol
MIC	Minimal inhibitory concentration
Mtt	4-Methoxytrityl
NMR	Nuclear magnetic resonance
PBS	Phosphate buffered saline
SPPS	Solid-phase peptide synthesis
TFA	Trifluoroacetic acid
TFE	2,2,2-Trifluoroethanol
TIS	Triisopropylsilane

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TLC	Thin-layer chromatography
t_R	Retention time
Trt	Trityl

Introduction

Peptides and proteins play ubiquitous key roles in many biological systems and have been recognized for their clinical potentials. At present, therapeutic peptides exist for a wide variety of human diseases (Stevenson 2009). However, despite the great potential, there are still some limitations for developing peptides as drugs per se. In particular, these limitations are high susceptibility to enzymatic proteolysis and poor penetration of biological barriers, leading to low oral bioavailability and short in vivo half-lives. For these reasons, efforts have been directed towards the synthesis of naturally occurring peptide mimics that display enhanced pharmacokinetic and biological properties (Liskamp et al. 2011). For instance, replacing a proteolytically cleavable peptide bond with bioisosteric [peptoids (Zuckermann et al. 1992; Fowler and Blackwell 2009; Yoo et al. 2010), azapeptides (Gante et al. 1995; Zega 2005), urea-peptidomimetics (Boeijen and Liskamp 1999), β-peptidosulfonamides (de Jong et al. 2002), depsipeptides (Cudic and Stawikowski 2007)] but less labile chemical entities afforded peptide analogs (pseudopeptides) with increased stability and biological activity. Other approaches have focused on the introduction of constrained amino acid mimetics that can imitate specific secondary structural features, conferring them with enhanced pharmacological properties (Toniolo 2004). The cyclization of biologically active peptides (Katsara et al. 2006; Karskela et al. 2006), the incorporation of non-proteinogenic amino acids (e.g. D-amino acids, *N*-alkylated amino acids, β-amino acids) (Gentilucci et al. 2010), as well as peptide glycosylation (Solá and Griebenow 2010) have also met success in improving the physicochemical and pharmacological properties of peptides.

Among the amino acid building blocks routinely employed in solid-phase peptide synthesis (SPPS), basic amino acids such as lysine, ornithine, diaminobutyric acid, and diaminopropionic acid have been extensively used for the synthesis of complex structures due to the reactivity of their side-chain primary amines (Fig. 1). For example, the availability of orthogonally protected lysine has been crucial in the synthesis of densely branched multiantigen peptide systems (MAPs) (Sebestik et al. 2011; Sadler and Tam 2002), constrained cyclic peptides (Tolle et al. 1982; Katsara et al. 2006; Zhang and Taylor 1996; Yu and Taylor 1996), α-helical bundle proteins (Hahn et al. 1990; Mutter et al. 1992), and collagen-like helical structures (Fields and Prockop 1996; Fields et al. 1993; Grab et al. 1996). In addition, the reactivity of the lysine side-chain amino group was also exploited during the preparation of neoglycoproteins through mono- and di-reductive amination using thioglycosides bearing an ω-aldehydoaglycon (Lee and Lee 1980). Interestingly, the acid hydrolysis of the obtained neoglycoprotein led to the identification of $N^{\varepsilon},N^{\varepsilon\prime}$-di(2-aminoethyl)-L-lysine.

The presence of basic amino acids has been also proved crucial for many cationic antimicrobial peptides (CAMPs) (Jenssen et al. 2006; Findlay et al. 2010) and cell-penetrating peptides (CPPs) (Green and Loewenstein 1988; Derossi et al. 1994; Oehlke et al. 1998). For the former, cationicity promotes the initial electrostatic interactions of the CAMP to the negatively charged phospholipid membranes of bacteria and other pathogens, conferring them with selectivity against microbes compared with host tissues. The presence of properly spaced positive charges has proved to be important for binding and neutralizing bacterial lipopolysaccharides (LPS), the primary cause of the Gram-negative septic shock (David 2001). A net positive charge at physiological pH has also been a key factor for the membrane translocation properties exhibited by CPPs (Futaki et al. 2001; Wender et al. 2000; Patel et al. 2007). Typically, the cationic CPPs contain clusters of primarily arginine and lysine residues.

Motivated by the importance and versatility of lysine and the growing needs for polyfunctional amino acid building blocks suitable for the synthesis of complex and diverse peptides on solid-phase, herein we report direct access to novel orthogonally protected *N*,*N*′-diaminoalkylated amino acid derivatives (Fig. 1). Our approach consists of the one-pot double reductive alkylation (Levadala et al. 2004; Bartholoma et al. 2009) of commercially available N^{α}-protected diamino acids (lysine, ornithine, 2,4-diaminobutyric acid, and 2,3-diaminopropionic acid) with convenient *N*-protected aliphatic amino aldehydes in the presence of sodium cyanoborohydride ($NaBH_3CN$) (Borch et al. 1971). The suitability of our synthetic basic amino acid building blocks possessing branched side-chains for utilization in SPPS has been demonstrated through the substitution of the sole lysine residue in the naturally occurring antimicrobial peptide indolicidin (ILPW**K**WPWWPWRR-NH_2) as a model peptide (Selsted et al. 1992). The effect of the substitution on the conformation, antibacterial activity, and human erythrocyte toxicity of the indolicidin analogue has also been described.

Materials and methods

General methods

Unless otherwise specified, all chemicals and solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and

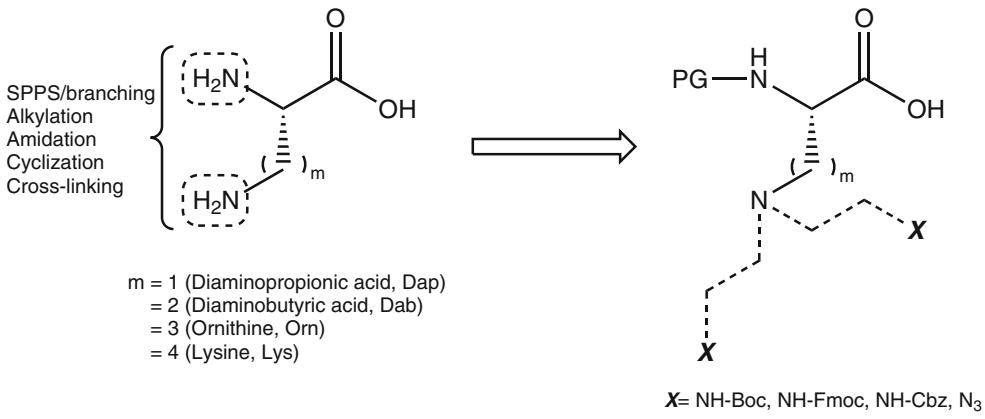


Fig. 1 Common applications of basic amino acids and design of novel *N*,*N*′-diaminoalkylated amino acid building blocks

were of analytical reagent grade or better. All amino acid precursors were purchased from Chem-Impex (Wood Dale, IL, USA) and used without further purification. Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanineamide, FDAA) was purchased from TCI America (Portland, OR, USA). 3-(*N*-benzyloxycarbonyl) aminopropionaldehyde was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The controlled addition of solutions was performed by a Chemyx® (Stafford, TX, USA) Fusion 200 syringe pump. Thin layer chromatography (TLC), used for monitoring reactions, was performed on Whatman precoated silica gel F-254 plates (Whatman Inc., Piscataway, NJ, USA) and visualized by ultraviolet light and/or staining with ninhydrin solution. Silica gel 60 (EMD Chemicals, Germany; particle size 0.040–0.063 mm, 230–400 Mesh ASTM) was used for flash chromatography. Reverse-phase flash chromatography was performed on a CombiFlash® Companion® using RediSep Rf Gold® C_{18} columns. The peptides were synthesized using a PS3 automated peptide synthesizer (Protein Technologies Inc., Tucson, AZ, USA). Mass spectrometric analyses (MS) of the amino acids and peptides were performed on MALDI–TOF Voyager-DE™ STR (Applied Biosystems, Foster City, CA, USA). The purity of our analogues and peptides (>95 %) was assessed on an analytical Agilent Infinity 1260 high-performance liquid chromatography (HPLC) system (Agilent Inc., Santa Clara, CA, USA) equipped with a UV/Vis detector. For reverse-phase HPLC analysis, a C_{18} monomeric column (Grace Vydac, 250 × 4.6 mm, 5 μm, 120 Å), 1 mL/min flow rate, and elution method of 98 % A for 0.5 min followed by linear gradient of 2–85 % B over 32 min were used, where A was 0.1 % TFA in H_2O and B was 0.08 % TFA in ACN. Eluting products were detected by UV at 214 and 267 nm. The enantiomeric integrity of the synthetic analogues was determined by derivatization with Marfey's reagent and HPLC analysis of the corresponding products. Rink amide 4-methylbenzhydrylamine (MBHA) resin (substitution level 0.66 mmol/g) was purchased from Novabiochem® (EMD Chemicals, NJ, USA). Circular dichroism (CD) spectra were recorded on a JASCO 810 spectropolarimeter (Easton, MD) using a quartz cell of 0.1 cm optical path length. Spectra were measured over a wavelength range of 180–250 nm with an instrument scanning speed 200 nm/min and a response time of 1 s. The concentrations of peptides were 0.125 mg/mL and 0.25 mg/mL. Microbial strains were purchased from American Type Culture Collection (ATCC, Manassas, VA). Dehydrated culture media and agar, and polystyrene plates used for antimicrobial assays were purchased from BD (Franklin Lakes, NJ). Control antibiotics were purchased from Sigma Aldrich (St. Louis, MO). Antimicrobial activity assays were carried out in standard, sterile 96-well plates, and minimal inhibitory concentration (MIC) values were determined by measuring turbidity at 600 nm using a Stat Fax 2100 Microplate reader (Awareness Technology Inc., Palm City, FL). Human red blood cells (hRBCs) were purchased from Innovative Research (Novi, MI). Human serum was purchased from Sigma Aldrich (St. Louis, MO).

NMR analysis

The ^{1}H and ^{13}C NMR spectra were recorded at 400 and 100 MHz, respectively, on a Bruker Avance 400 MHz NMR spectrometer (Billerica, MA). Samples were prepared in tubes with a diameter of 5 mm employing 0.5 mL of the indicated deuterated solvent. The chemical shifts (δ) are reported in units of parts per million (ppm) downfield from tetramethylsilane. The assignment of the ^{1}H NMR signals was based on ^{1}H-^{1}H and ^{13}C-^{1}H correlation spectrometry.

General procedure for the double reductive alkylation of N^{α}-protected diamino acids

A solution of N^{α}-protected diamino acid (**1**–**4**) in MeOH (90 mL/mmol) containing 1–6 % AcOH was treated with a methanolic solution of the corresponding amino aldehyde (3.0 eq.). After stirring for 1 h at room temperature, a

solution of $NaBH_3CN$ in MeOH (1 mL) was added dropwise for 2 h. The resulting clear solution was stirred until TLC and MALDI-TOF MS analyses of representative aliquots confirmed the complete conversion to the desired di-alkylated building block. The reaction mixture was concentrated by rotary evaporation and mixed with EtOAc before washing with water. The organic phase was separated and the aqueous layer extracted three times with fresh EtOAc. The combined organics were dried over anhydrous sodium sulfate, filtered, and evaporated under vacuum. The crude residues were purified by flash chromatography (silica gel, DCM/MeOH/AcOH 85:10:5) or reverse-phase flash chromatography (C-18 silica gel, 5–85 % gradient of ACN in H_2O containing 0.1 FA) to give the following pure di-alkylated products (**5**–**15**):

N^{α}-(9*H*-fluorenyl-9-ylmethoxycarbonyl)-N^{β},$N^{\beta\prime}$-bis[2-(*tert*-butoxycarbonylamino)ethyl]-L-2,3-diaminopropionic acid **5**. HPLC: $t_R = 26.272$ min; ^{1}H NMR (400 MHz, Methanol-d_4) δ 7.81 (d, $J = 7.5$ Hz, 2H, Fmoc-*H*), 7.67 (d, $J = 7.4$ Hz, 2H, Fmoc-*H*), 7.40 (t, $J = 7.4$ Hz, 2H, Fmoc-*H*), 7.31 (t, $J = 7.4$ Hz, 2H, Fmoc-*H*), 4.69 (dd, $J = 8.6$, 5.3 Hz, 1H, Dap-H^{α}), 4.47 (dd, $J = 10.4$, 7.1 Hz, 1H, Fmoc-CH_{2a}), 4.37 (dd, $J = 10.2$, 7.2 Hz, 1H, Fmoc-CH_{2b}), 4.25 (t, $J = 6.9$ Hz, 1H, Fmoc-C*H*), 3.81 (dd, $J = 13.6$, 5.3 Hz, 1H, Dap-H_1^{β}), 3.57 (dd, $J = 13.5$, 9.1 Hz, 1H, Dap-H_2^{β}), 3.44 (s, 8H, arms-CH_2), 1.43 (s, 18H, Boc-CH_3); ^{13}C NMR (101 MHz) δ 171.81, 159.34, 158.83, 145.05, 142.62, 128.89, 128.19, 126.21, 121.00, 81.48, 68.59, 56.25, 50.88, 36.73, 28.68; MALDI-TOF MS, calculated *m*/*z* 612.71, found *m*/*z* 613.94 $[M + H]^+$, 635.92 $[M + Na]^+$.

N^{α}-(9*H*-fluorenyl-9-ylmethoxycarbonyl)-N^{γ},$N^{\gamma\prime}$-bis[2-(*tert*-butoxycarbonylamino)ethyl]-L-2,4-diaminobutyric acid **6**. HPLC: $t_R = 25.679$ min; ^{1}H NMR (400 MHz, Methanol-d_4) δ 7.79 (d, $J = 7.4$ Hz, 2H, Fmoc-*H*), 7.66 (t, $J = 6.3$ Hz, 2H, Fmoc-*H*), 7.39 (t, $J = 7.4$ Hz, 2H, Fmoc-*H*), 7.31 (t, $J = 7.3$ Hz, 2H, Fmoc-*H*), 4.37 (d, $J = 6.7$ Hz, 2H, Fmoc-CH_2), 4.23 (t, $J = 6.7$ Hz, 1H, Fmoc-C*H*), 4.13 (t, $J = 6.3$ Hz, 1H, Dab-H^{α}), 3.40–3.30 (m, 4H, arms-CH_2), 3.22–2.88 (m, 6H, Dab-H_2^{γ} + arms-CH_2), 2.25–2.07 (m, 1H, Dab-H_1^{β}), 2.04–1.95 (m, 1H, Dab-H_2^{β}), 1.43 (s, 18H, Boc-CH_3); ^{13}C NMR (101 MHz) δ 158.61, 158.36, 145.37, 142.63, 128.81, 128.18, 126.21, 120.96, 80.80, 67.99, 55.17, 54.73, 53.12, 48.47, 28.75, 20.95; MALDI-TOF MS, calculated *m*/*z* 626.74, found *m*/*z* 627.72 $[M + H]^+$.

N^{α}-(9*H*-fluorenyl-9-ylmethoxycarbonyl)-N^{δ},$N^{\delta\prime}$-bis[2-(*tert*-butoxycarbonylamino)ethyl]-L-ornithine **7**. HPLC: $t_R = 25.742$ min; ^{1}H NMR (400 MHz, Methanol-d_4) δ 7.79 (d, $J = 7.5$ Hz, 2H, Fmoc-*H*), 7.66 (dd, $J = 11.0$, 7.6 Hz, 2H, Fmoc-*H*), 7.39 (t, $J = 7.4$ Hz, 2H, Fmoc-*H*), 7.31 (t, $J = 7.4$ Hz, 2H, Fmoc-*H*), 4.40 (dd, $J = 10.4$, 6.7 Hz, 1H, Fmoc-CH_{2a}), 4.30 ("t", $J = 8.9$ Hz, 1H, Fmoc-CH_{2b}), 4.21 (t, $J = 6.8$ Hz, 1H, Fmoc-C*H*), 4.08 (d, $J = 6.5$ Hz, 1H, Orn-H^{α}), 3.39–3.32 (m, 4H, arms-CH_2), 3.11 (br.s, 6H, Orn-H_2^{γ} + arms-CH_2), 1.86 (t, $J = 6.7$ Hz, 1H, Orn-H_1^{β}), 1.74 (br.s, 3H, Orn-H_2^{β} + Orn-H_2^{γ}), 1.40 (s, 18H, Boc-CH_3); ^{13}C NMR (101 MHz) δ 176.04, 159.10, 158.60, 145.37, 145.15, 142.61, 128.83, 128.81, 128.19, 128.16, 126.31, 126.15, 120.95, 81.23, 68.06, 62.05, 55.37, 55.13, 54.39, 36.98, 30.22, 28.71, 21.77; MALDI-TOF MS, calculated *m*/*z* 640.77, found *m*/*z* 641.61 $[M + H]^+$, 663.59 $[M + Na]^+$.

N^{α}-(9*H*-fluorenyl-9-ylmethoxycarbonyl)-N^{ε},$N^{\varepsilon\prime}$-bis[2-(*tert*-butoxycarbonylamino)ethyl]-L-lysine **8**. HPLC: $t_R = 25.720$ min; ^{1}H NMR (400 MHz, Methanol-d_4) δ 7.79 (d, $J = 7.3$ Hz, 2H, Fmoc-*H*), 7.67 (t, $J = 7.8$ Hz, 3H, Fmoc-*H*), 7.39 (t, $J = 7.3$ Hz, 3H, Fmoc-*H*), 7.30 (t, $J = 7.2$ Hz, 3H, Fmoc-*H*), 4.40 (dd, $J = 10.2$, 5.1 Hz, 1H, Fmoc-CH_{2a}), 4.31 (dd, $J = 8.8$, 4.2 Hz, 1H, Fmoc-CH_{2b}), 4.22 (t, $J = 7.5$ Hz, 1H, Fmoc-C*H*), 4.20–4.14 (m, 1H, Lys-H^{α}), 3.41 (d, $J = 5.5$ Hz, 4H, arms-CH_2), 3.35–3.26 (m, 4H, arms-CH_2), 3.26–3.15 (m, 2H, Lys-H_2^{ε}), 1.98–1.84 (m, 1H, Lys-H_1^{β}), 1.84–1.63 (m, 3H, Lys-H_2^{β} + Lys-H_2^{δ}), 1.44 (s, 20H, Boc-CH_3 + Lys-H_2^{γ}); ^{13}C NMR (101 MHz) δ 175.45, 159.22, 158.71, 145.33, 145.15, 142.60, 128.81, 128.18, 128.15, 126.26, 126.17, 120.94, 81.39, 67.99, 55.38, 54.78, 54.69, 36.72, 32.20, 28.69, 24.23, 23.85; MALDI-TOF MS, calculated *m*/*z* 654.79, found *m*/*z* 656.00 $[M + H]^+$.

N^{α}-(*tert*-butoxycarbonyl)-N^{β},$N^{\beta\prime}$-bis[2-(9*H*-fluorenyl-9-ylmethoxycarbonylamino)ethyl]-L-2,3-diaminopropionic acid **9**. HPLC: $t_R = 29.495$ min; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.87 (d, $J = 7.3$ Hz, 4H, Fmoc-*H*), 7.66 (d, $J = 7.4$ Hz, 4H, Fmoc-*H*), 7.39 (t, $J = 7.4$ Hz, 4H, Fmoc-*H*), 7.31 (t, $J = 7.4$ Hz, 4H, Fmoc-*H*), 7.20 (t, $J = 5.7$ Hz, 2H, Fmoc-CO–N*H*), 6.94 (d, $J = 7.7$ Hz, 1H, Boc- CO–N*H*), 4.27 (d, $J = 7.0$ Hz, 4H, Fmoc-CH_2), 4.19 (t, $J = 6.9$ Hz, 2H, Fmoc-C*H*), 4.03 ("q", $J = 7.5$ Hz, 1H, Dap-H^{α}), 3.05 ("q", $J = 6.6$ Hz, 4H, arms-CH_2), 2.80 (dd, $J = 13.7$, 5.7 Hz, 1H, Dap-H_1^{β}), 2.72 (dd, $J = 13.3$, 8.1 Hz, 1H, Dap-H_2^{β}), 2.64–2.51 (m, 4H, arms-CH_2), 1.34 (s, 9H, Boc-CH_3); ^{13}C NMR (101 MHz) δ 173.22, 163.20, 156.23, 155.43, 143.91, 143.87, 140.71, 127.60, 127.06, 125.16, 120.09, 78.12, 65.45, 54.82, 53.29, 52.19, 46.76, 38.31, 28.17; MALDI-TOF MS, calculated *m*/*z* 734.84, found *m*/*z* 735.99 $[M + H]^+$, 757.99 $[M + Na]^+$.

N^{α}-(*tert*-butoxycarbonyl)-N^{ε},$N^{\varepsilon\prime}$-bis[3-(9*H*-fluorenyl-9-ylmethoxycarbonylamino)propyl]-L-lysine **10**. HPLC: $t_R = 28.586$ min; ^{1}H NMR (400 MHz, Methanol-d_4) δ 7.76 (d, $J = 7.5$ Hz, 4H, Fmoc-*H*), 7.60 (d, $J = 7.6$ Hz, 4H, Fmoc-*H*), 7.36 (t, $J = 7.5$ Hz, 4H, Fmoc-*H*), 7.28 (t, $J = 7.2$ Hz, 4H, Fmoc-*H*), 4.37 (d, $J = 6.7$ Hz, 4H, Fmoc-

CH_2), 4.16 (t, $J = 6.8$ Hz, 2H, Fmoc-C*H*), 4.10 (dd, $J = 8.4$, 4.5 Hz, 1H, Lys-H^{α}), 3.18 (t, $J = 6.2$ Hz, 4H, arms-CH_2), 3.14–2.96 (m, 6H, arms-CH_2 + Lys-H_2^{ε}), 1.92–1.76 (m, 5H, Lys-H_1^{β} + arms-CH_2-CH_2-CH_2), 1.74–1.59 (m, 3H, Lys-H_2^{β} + Lys-H_2^{δ}), 1.42 (s, 11H, Boc-CH_3 + Lys-H_2^{γ}); ^{13}C NMR (101 MHz) δ 175.74, 159.19, 158.13, 145.22, 142.63, 128.81, 128.74, 128.12, 126.02, 120.98, 80.63, 67.73, 54.32, 53.87, 51.78, 38.60, 32.30, 28.73, 25.51, 24.06, 23.85; MALDI-TOF MS, calculated m/z 804.97, found m/z 806.05 $[M + H]^+$.

N^{α}-(9*H*-fluorenyl-9-ylmethoxycarbonyl)-N^{β},$N^{\beta\prime}$-bis[3-(benzyloxycarbonylamino)propyl]-L-2,3-diaminopropionic acid **11**. HPLC: $t_R = 26.577$; ^{1}H NMR (400 MHz, Methanol-d_4) δ 7.77 (d, $J = 7.4$ Hz, 2H, Fmoc-*H*), 7.63 (t, $J = 8.1$ Hz, 2H, Fmoc-*H*), 7.37 (t, $J = 7.3$ Hz, 2H, Fmoc-*H*), 7.28 (dd, $J = 9.0$, 5.6 Hz, 12H, Fmoc-*H* + Cbz-*H*), 5.02 (s, 4H, Cbz-CH_2), 4.36 (dd, $J = 6.9$, 3.6 Hz, 2H, Fmoc-CH_2), 4.30 (t, $J = 7.6$ Hz, 1H, Dap-H^{α}), 4.19 (t, $J = 6.6$ Hz, 1H, Fmoc-C*H*), 3.28–3.22 (m, 1H, Dap-H_1^{β}), 3.24–3.03 (m, 9H, Dap-H_2^{β} + arms-CH_2), 1.87 (s, 4H, arms-CH_2-CH_2-CH_2); ^{13}C NMR (101 MHz, MeOD) δ 174.38, 159.06, 158.49, 145.28, 145.12, 142.61, 142.58, 138.25, 129.48, 129.02, 128.82, 128.16, 126.19, 120.95, 68.28, 67.61, 60.39, 55.77, 52.68, 50.95, 38.70, 25.70; MALDI-TOF MS, calculated m/z 708.80, found m/z 709.99 $[M + H]^+$, 731.98 $[M + Na]^+$.

N^{α}-(9*H*-fluorenyl-9-ylmethoxycarbonyl)-N^{ε},$N^{\varepsilon\prime}$-bis[3-(benzyloxycarbonylamino)propyl]-L-lysine **12**. HPLC: $t_R = 26.333$ min; ^{1}H NMR (400 MHz, Methanol-d_4) δ 7.79 (d, $J = 7.5$ Hz, 2H, Fmoc-*H*), 7.66 (dd, $J = 9.8$, 7.6 Hz, 2H, Fmoc-*H*), 7.38 (t, $J = 7.5$ Hz, 2H, Fmoc-*H*), 7.35–7.23 (m, 12H, Fmoc-*H* + Cbz-*H*), 5.06 (s, 4H, Cbz-CH_2), 4.40 (dd, $J = 10.4$, 6.8 Hz, 1H, Fmoc-CH_{2a}), 4.32 (dd, $J = 10.5$, 7.0 Hz, 1H, Fmoc-CH_{2b}), 4.21 (t, $J = 6.6$ Hz, 1H, Fmoc-C*H*), 4.21–4.15 (m, 1H, Lys-H^{α}), 3.19 (t, $J = 6.4$ Hz, 4H, arms-CH_2), 3.10 (t, $J = 8.1$ Hz, 4H, arms-CH_2), 3.11–2.96 (m, 2H, Lys-H_2^{ε}), 1.91–1.78 (m, 5H, Lys-H_1^{β} + arms-CH_2-CH_2-CH_2), 1.78–1.55 (m, 3H, Lys-H_2^{β} + Lys-H_2^{δ}), 1.51–1.35 (m, 2H, Lys-H_2^{γ}); ^{13}C NMR (101 MHz) δ 175.69, 159.20, 158.66, 145.33, 145.13, 142.58, 138.21, 129.51, 129.09, 128.84, 128.81, 128.18, 128.15, 126.25, 126.15, 120.96, 67.96, 67.69, 54.83, 53.81, 51.73, 38.63, 32.23, 25.52, 23.88, 23.79; MALDI-TOF MS, calculated m/z 750.88, found m/z 752.08 $[M + H]^+$.

N^{α}-(*tert*-butoxycarbonyl)-N^{β},$N^{\beta\prime}$-bis[3-(benzyloxycarbonylamino)propyl]-L-2,3-diaminopropionic acid **13**. HPLC: $t_R = 22.678$ min; ^{1}H NMR (400 MHz, Methanol-d_4) δ 7.39–7.25 (m, 10H, Cbz-*H*), 5.07 (s, 4H, Cbz-CH_2), 4.27 (t, $J = 6.8$ Hz, 1H, Dap-H^{α}), 3.31–3.28 (m, 2H, Dap-H_2^{β}), 3.22 (t, $J = 6.3$ Hz, 8H, arms-CH_2), 1.91 (p, $J = 8.4$, 7.6 Hz, 4H, arms-CH_2-CH_2-CH_2), 1.45 (s, 9H, Boc-CH_3). ^{13}C NMR (101 MHz) δ 174.36, 159.04, 157.97, 138.27, 129.49, 129.04, 128.83, 81.27, 67.61, 55.97, 52.82, 50.61, 38.73, 28.66, 25.69; MALDI-TOF MS, calculated m/z 586.68, found m/z 587.71 $[M + H]^+$.

N^{α}-(benzyloxycarbonyl)-N^{β},$N^{\beta\prime}$-bis[2-(*tert*-butoxycarbonylamino)ethyl]-L-2,3-diaminopropionic acid **14**. HPLC: $t_R = 22.377$ min; ^{1}H NMR (400 MHz, Methanol-d_4) δ 7.42–7.25 (m, 5H, Cbz-*H*), 5.11 (s, 2H, Cbz-CH_2), 4.27 (t, $J = 7.2$ Hz, 1H, Dap-H^{α}), 3.29 (br. s, 4H, arms-CH_2), 3.25–3.20 (m, 2H, Dap-H_2^{β}), 3.18–2.99 (m, 4H, arms-CH_2), 1.43 (s, 18H, Boc-CH_3). ^{13}C NMR (101 MHz) δ 174.80, 158.53, 158.48, 138.06, 129.52, 129.09, 128.97, 80.67, 67.92, 57.30, 54.96, 52.10, 37.80, 28.81; MALDI-TOF MS, calculated m/z 524.61, found m/z 525.90 $[M + H]^+$.

N^{α}-(9*H*-fluorenyl-9-ylmethoxycarbonyl)-N^{β},$N^{\beta\prime}$-bis(3-azidopropyl)-L-2,3-diaminopropionic acid **15**. HPLC: $t_R = 23.570$ min; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.89 (d, $J = 7.4$ Hz, 2H, Fmoc-*H*), 7.71 (dd, $J = 7.4$, 2.9 Hz, 2H, Fmoc-*H*), 7.57 (d, $J = 8.4$ Hz, 1H, Fmoc-CO–N*H*), 7.42 (t, $J = 7.3$ Hz, 2H, Fmoc-*H*), 7.32 (td, $J = 7.4$, 1.2 Hz, 2H, Fmoc-*H*), 4.29 (dd, $J = 7.0$, 3.6 Hz, 2H, Fmoc-CH_2), 4.23 (q, $J = 6.9$, 6.1 Hz, 1H, Fmoc-C*H*), 4.12 (td, $J = 8.2$, 5.9 Hz, 1H, Dap-H^{α}), 3.32 (t, $J = 6.7$ Hz, 4H, arms-CH_2), 2.76 (dd, $J = 13.3$, 6.1 Hz, 1H, Dap-H_1^{β}), 2.66 (dd, $J = 13.3$, 8.1 Hz, 1H, Dap-H_2^{β}), 2.53–2.38 (m, 4H, arms-CH_2), 1.70–1.54 (m, 4H, arms-CH_2-CH_2-CH_2); ^{13}C–NMR (101 MHz) δ 172.96, 155.90, 143.83, 143.78, 140.73, 127.63, 127.03, 125.21, 120.10, 65.70, 54.59, 52.37, 50.45, 48.71, 46.66, 25.79; MALDI-TOF MS, calculated m/z 492.53, found m/z 493.77 $[M + H]^+$, 515.75 $[M + Na]^+$.

One-pot synthesis of unsymmetrically N,N'-diaminoalkylated amino acid derivative **17** via tandem double reductive alkylation.

A solution of **1a** (100 mg, 0.31 mmol) in MeOH containing 6 % AcOH (32 mL) was treated simultaneously with methanolic solutions of 2-(*N*-tertbutoxycarbonyl)aminoacetaldehyde (59 mg, 0.37 mmol) and $NaBH_3CN$ (16 mg, 0.25 mmol) for 4 h. Upon stirring at room temperature for additional 4 h, solutions of 3-azidopropionaldehyde (46 mg, 0.46 mmol) and $NaBH_3CN$ (16 mg, 0.25 mmol) in MeOH were added simultaneously for 4 h and the reaction mixture stirred overnight at room temperature. The reaction mixture was concentrated by rotary evaporation and mixed with EtOAc before washing with water. The organic phase was separated and the aqueous layer extracted 3 times with EtOAc. The combined organic layers were dried over anhydrous sodium sulfate, filtered, and evaporated under vacuum. HPLC of the crude reaction mixture revealed the presence of three major products at t_R 23.57, 25.01, and 26.27 min. The mixture of products was

separated by reverse-phase flash chromatography (5–85 % gradient of ACN in H_2O, both containing 0.1 % FA) to give pure unsymmetrically substituted product **17** (65 mg, 38 %) as a white powder.

N^{α}-(9*H*-fluorenyl-9-ylmethoxycarbonyl)-N^{β}-(3-azidopropyl)-$N^{\beta\prime}$-(2-(*tert*-butoxycarbonylamino)-ethyl)-L-2,3-diaminopropionic acid **17**. HPLC: $t_R = 25.010$ min; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.89 (d, $J = 7.5$ Hz, 2H, Fmoc-*H*), 7.72 (d, $J = 7.4$ Hz, 2H, Fmoc-*H*), 7.58 (d, $J = 8.0$ Hz, 1H, Fmoc-CO–N*H*), 7.41 (t, $J = 7.4$ Hz, 2H, Fmoc-*H*), 7.32 (t, $J = 7.4$ Hz, 2H, Fmoc-*H*), 6.64 (t, $J = 5.4$ Hz, 1H, Boc-CO–N*H*), 4.32–4.17 (m, 3H, Fmoc-CH_2 + Fmoc-C*H*), 4.11 (q, $J = 7.8$ Hz, 1H, Dap-H^{α}), 3.32 (t, $J = 6.8$ Hz, 2H, arm-CH_2–N_3), 2.99 (q, $J = 6.7$ Hz, 2H, arm-CH_2–NHBoc), 2.79 (dd, $J = 13.4$, 6.1 Hz, 1H, Dap-H^{β}_1), 2.71 (dd, $J = 13.3$, 8.3 Hz, 1H, Dap-H^{β}_2), 2.60–2.40 (m, 4H, arm-CH_2–N_3 + arm-CH_2–NHBoc), 1.61 (dq, $J = 13.4$, 6.8 Hz, 2H, arm-CH_2–CH_2–CH_2–N_3), 1.35 (s, 9H, Boc-CH_3); ^{13}C NMR (101 MHz) δ 172.93, 155.97, 155.64, 143.80, 143.76, 140.70, 127.62, 127.04, 125.27, 125.24, 120.09, 77.57, 65.77, 54.79, 53.09, 52.37, 50.33, 48.69, 46.63, 37.72, 28.20, 25.93; MALDI-TOF MS, calculated *m/z* 552.62, found *m/z* 553.85 $[M + H]^+$.

Synthesis of indolicidin and its modified analogue **Indo-5**

Both indolicidin and its analogue **Indo-5** were synthesized using Fmoc-protected amino acids on a Rink amide 4-methylbenzhydrylamine (MBHA) resin (substitution level 0.66 mmol/g) by solid-phase Fmoc- methodology on a PS3 automated peptide synthesizer. Cleavage of the peptides from the solid support was carried out by Reagent K containing TIS (TFA/H_2O/thioanisole/phenol/EDT/TIS 81.5:5:5:5: 2.5:1 v/v) for 2 h at room temperature. The crude peptides were precipitated, washed with cold methyl *tert*-butyl ether, and purified on an Agilent Infinity 1260 HPLC system using a preparative Vydac C_{18} column (Grace Vydac, 250 × 22 mm, 10 µm, 120 Å) with an elution method consisting of a linear gradient of 2–85 % B over 32.5 min, where A was 0.1 % TFA in H_2O and B was 0.08 % TFA in ACN. The final purity of the peptides (>98 %) was assessed by analytical reverse-phase HPLC using conditions described in the general methods. The molecular masses were determined using MALDI-TOF MS.

Antimicrobial activity

The assessment of antibacterial activity and determination of MIC values was done using standard micro dilution broth method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2011) using 96-well flat-bottomed polystyrene microtiter plates. Plates were loaded with 90 µL of mid-logarithmic phase cells with initial OD_{600} 0.001 of the tested microorganism and 10 µL aliquots of twofold serial dilutions of the control antibiotics or tested peptide analogues. Controls on each plate were media without bacteria, bacterial inoculum without antimicrobials added and bacterial inoculum to which control antibiotics were added. In the case of Gram-positive bacteria controls were methicillin and vancomycin and in the case of Gram-negative bacteria ampicillin and ciprofloxacin. Concentrations of these antibiotics were the same as of the tested peptides, in the range from 1 to 128 µg/mL. All samples were loaded in duplicates. Plates were incubated at 37 °C overnight with gentle shaking. The inhibition of the bacterial growth was determined by measuring OD_{600}.

Hemolytic activity

Human red blood cells (hRBCs) were diluted with PBS to 2 %. Into each well of the clear, flat-bottom 96-well plate, 50 µL of the hRBCs was placed followed by addition of 50 µL of peptide solution to final peptide concentrations of 8–64 µg/mL. Assays were performed in triplicate, and each experiment was repeated twice. As a positive control, 50 µL of Triton X-100 in water was used at a final concentration of 0.5 % (v/v). As a negative control, 50 µL of water and PBS was used. Plates were incubated for 1 h at 37 °C after which 100 µL of PBS was added to each well, and the plates were centrifuged for 10 min at 1,000*g*. Supernatants (150 µL) were transferred into a new plate, and absorbance at 405 nm was measured. The data were corrected for background to obtain solely hemolytic activity of the peptides. The degree of hemolysis was expressed in percent relative to total hemolysis caused by Triton-X-100.

Results and discussion

The main goal of the work described here was to synthesize novel orthogonally protected branched basic amino acids suitable for the construction of complex peptides on a solid support. The versatility and general applicability of the described synthetic route were demonstrated by utilization of a variety of amino acid and aldehyde starting materials, Table 1. In order to meet the requirements of standard solid-phase peptide synthesis, Fmoc, Boc, Cbz carbamates, and azide (Lundquist and Pelletier 2001) as amine protecting groups were used.

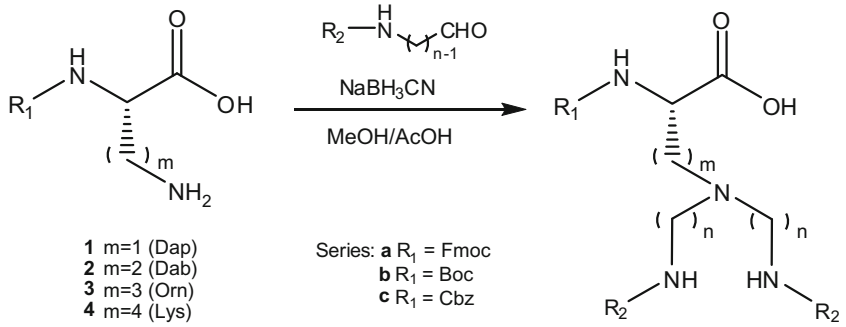
The treatment of N^{α}-Fmoc-2,3-diaminopropionic acid (**1a**) with excess 2-(*N-tert*-butoxycarbonyl) aminoacetaldehyde (3 eq.) in the presence of $NaBH_3CN$ afforded the protected dialkylated amino acid **5** in 85 % yield (entry 1,

Table 1). The acidic conditions employed (6 % AcOH in MeOH, pH ~4) were optimal for the complete solubilization of the diamino acid precursor and the stability of the reducing agent. Moreover, the acid-labile Boc-protecting group displayed complete stability after prolonged stirring and post-reaction manipulations. Similar treatment of longer, offering less sterically hindered reaction sites, N^{α}-Fmoc amino acid precursors **2a**–**4a** resulted in the corresponding branched basic amino acids **6**–**8** in excellent yields and purities (entries 2–4). The direct use of ornithine derivative **3a** as a hydrochloric salt did not affect the reaction outcome under the optimized conditions. Employing the same approach, the synthesis of analogues bearing a N^{α}-Boc protecting group was explored as well. As in the aforementioned cases, no steric effect on the reaction yield was observed. The reaction of **4b** with 3-(*N*-9-fluorenylmethoxycarbonyl) aminopropionaldehyde (Reggelin et al. 2006) resulted in 89 % yield of the desired product **10** (entry 6). Shortening the length of both building blocks, **1b** and 2-(*N*-9-fluorenylmethoxycarbonyl) aminoacetaldehyde (Diness et al. 2004) did not affect the reaction outcome, and product **9** was obtained in 80 % yield (entry 5).

The orthogonality of both Fmoc and Boc protecting groups present in analogues **5**–**10** allows the modification of the branched side-chains while on solid support, a desirable feature during the construction of complex peptides. However, the acidic conditions required for the removal of Boc limit the use of more acid labile protecting groups (e.g. Trt or Mtt) and acid labile resins (e.g. trityl, Sieber amide, Rink acid). Also, it is worth noting that the incorporation of a second side-chain containing primary amine offers the possibility of exploiting such branched amino acid building blocks as lysine mimics bearing two positive charges under physiological conditions (vide infra).

Therefore, it was important to demonstrate that the described reaction conditions are compatible with other combinations of orthogonal protecting groups. The reaction of N^{α}-Fmoc-protected substrates **1a** and **4a** with 3-(*N*-benzyloxycarbonyl) aminopropionaldehyde yielded the corresponding dialkylated products **11** and **12** in 86 and

Table 1 Double reductive alkylation of N^{α}-protected diamino acids with selected *N*-protected amino aldehydes



Entry	Substrate	R_1	*n*	R_2	Product	Yield (%)[a]
1	**1a**	Fmoc	2	Boc	**5**	85
2	**2a**	Fmoc	2	Boc	**6**	89
3	**3a**	Fmoc	2	Boc	**7**	87
4	**4a**	Fmoc	2	Boc	**8**	88
5	**1b**	Boc	2	Fmoc	**9**	80
6	**4b**	Boc	3	Fmoc	**10**	89
7	**1a**	Fmoc	3	Cbz	**11**	86
8	**4a**	Fmoc	3	Cbz	**12**	89
9	**1b**	Boc	3	Cbz	**13**	82
10	**1c**	Cbz	2	Boc	**14**	81
11	**1a**	Fmoc	3	N_3	**15**	65[b]

The protected diamino acid dissolved in MeOH/AcOH was treated with the corresponding aldehyde (3.0 eq.) followed by a solution of $NaBH_3CN$ in MeOH and stirring at room temperature

[a] starting diamino acid as limiting reagent

[b] purified by RP-flash chromatography with a gradient 5–100 % B, where A was 0.1 % FA in H_2O and B was 0.1 % FA in ACN

89 %, respectively (entries 7 and 8). Identical treatment of N^{α}-Boc-2,3-diaminopropionic acid **1b** with excess 3-(*N*-benzyloxycarbonyl) aminopropionaldehyde gave compound **13** in 82 % yield (entry 9). No effect on the reaction yield was observed upon switching the protecting groups of starting materials. The reaction of N^{α}-Cbz-Dap-OH **1c** with 2-(*N-tert*-butoxycarbonyl) aminoacetaldehyde afforded analogue **14** in 81 % yield and high purity. The stability displayed by Cbz protecting group towards the conditions commonly employed in Fmoc SPPS, makes analogues **11** and **12** suitable for the construction of peptides that can be further modified upon release of the protected peptide from hyperacid labile resins (e.g. Rink acid, HMPB, 2-chlorotrityl) or resins susceptible to nucleophilic displacement (e.g. HMBA) (White and Chan 2003; Novabiochem Catalog 2010/2011; Tsubery et al. 2000; White and Yudin 2011). However, taking into consideration that the solid-phase methodology is a standard approach for routine peptide synthesis, modifications of peptide's basic amino acid sequence(s) on the solid support is highly desirable. The unique and versatile properties offered by the azide group made it a very attractive option for functionalization of the poly-amino acid building blocks that will allow further solid-phase modifications.

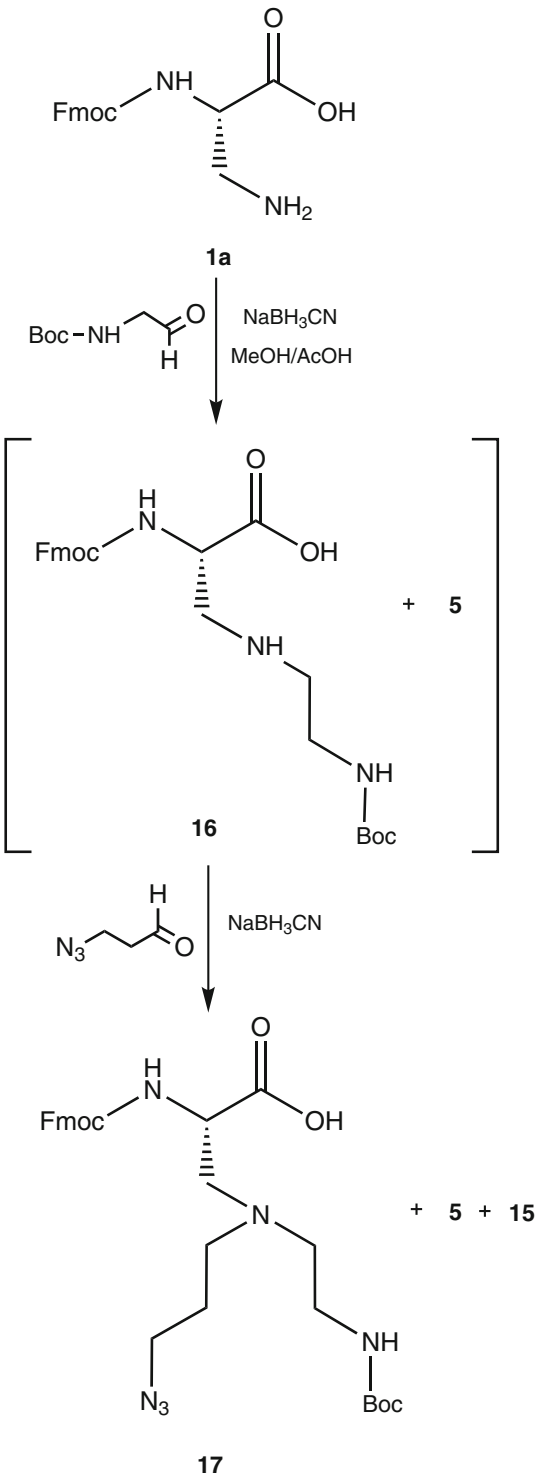
For example, the azide group can be considered as a temporarily protected amine that can be selectively transformed into a free amine by treatment with an adequate aryl/alkyl phosphine in the presence of water (Lundquist and Pelletier 2002). Also, efficient conditions have been described for the direct conversion of azides to various carbamates, including allyloxycarbamate (Aloc) (Ariza et al. 1999). More importantly, organic azides have been increasingly popular for their chemoselective and mild reaction with terminal alkynes via 1,3-dipolar cycloaddition (Hüisgen reaction) in the presence of copper (I) salts (Rostovtsev et al. 2002; Tornøe et al. 2002). The extraordinary level of selectivity, reliability, simplicity, and scope displayed by this reaction place it as a gold standard in the field of 'click chemistry' (Kolb et al. 2001). Furthermore, modifications of this reaction in which the toxic copper is not necessary have been reported to be highly compatible in the study of biological processes in living systems (Chang et al. 2010; Ning et al. 2008; Lutz 2008). Other applications of azides in organic synthesis have been reviewed as well (Bräse et al. 2005). Taking all these into consideration, we have synthesized symmetrical diazido **15** as well as unsymmetrical monoazido **17** amino acid derivatives.

Symmetrical derivative **15** was synthesized by controlled addition of 3-azidopropionaldehyde (3 eq.) (Boyer 1951; Davies et al. 1967) and $NaBH_3CN$ to a solution of **1a** in MeOH/AcOH (65 % yield, entry 11). In an attempt to synthesize unsymmetrically substituted amino acid building blocks in a single step, a solution of **1a** in MeOH/AcOH was treated simultaneously with methanolic solutions of 2-(*N-tert*-butoxycarbonyl)aminoacetaldehyde (1.2 eq.) and $NaBH_3CN$ (0.8 eq.) for a period of 4 h (Scheme 1). Upon stirring at room temperature for additional 4 h, the presence of monoalkylated derivative **16** along with unreacted starting **1a** and homo-dialkylated **5** was confirmed by TLC. Consequently, the simultaneous addition of 3-azidopropionaldehyde (1.5 eq.) and a second portion of $NaBH_3CN$ (0.8 eq.) for 4 h yielded a mixture of the desired mixed-dialkylated product **17** (52 %) along with homo-dialkylated byproducts **5** (25 %) and **15** (22 %) (based on RP-HPLC analysis of the crude reaction mixture). The mixture of products **5**, **15**, and **17** (t_R 26.27, 23.57, and 25.01 min) was efficiently separated by reverse-phase flash chromatography to give unsymmetrically substituted product **17** in overall 38 % yield. Although the synthesis of mixed dialkylated amino acid building blocks has been previously reported (Levadala et al. 2004), the nature of the appended groups (heterocyclic, carboxylic acid) in described derivatives prevents their further modification upon incorporation into the peptide sequences. In contrast, branched amino acid-building blocks **15** and **17** are fully compatible with the standard Boc and Fmoc-SPPS allowing their further synthetic exploitation in preparation of diverse and unusual peptide sequences.

It has been reported previously that activation of carboxylic acids by dialkoxyboranes may result in the formation of amides and lactams (Collum et al. 1978). Also, the tandem amination-lactamization of ortho-carboxybenzaldehyde with amines and amino esters in the presence of $NaBH(OAc)_3$ has been suggested to take place via carboxyl group activation by a borane or borate intermediate (Abdel-Magid et al. 1994). However, in our case no cyclic byproducts were detected under the applied reaction conditions. In addition, the stereochemical integrity of diamino acids **1**–**4** was not compromised by the alkylation reactions, as indicated by RP-HPLC analysis of product **6** as a model system, upon derivatization with Marfey′s reagent (Bhushan and Brückner 2004) (Online Resource).

Synthesis of modified indolicidin analogues

In order to test the suitability of synthesized branched basic amino acid building blocks for use in standard automated Fmoc-solid-phase peptide synthesis, we have prepared an analogue of the cationic antimicrobial peptide indolicidin, as a model peptide, in which its sole lysine residue was replaced with *N*,*N*′-diaminoalkylated lysine analogue **5** (**Indo-5**). Indolicin (H-Ile-Leu-Pro-Trp-Lys-Trp-Pro-Trp–Trp-Pro-Trp-Arg–Arg-NH_2) is a 13 amino acid *C*-terminus amidated peptide isolated from the granules of bovine neutrophils and it is the smallest known, naturally



Scheme 1 One-pot synthesis of unsymmetrically substituted *N*,*N*′-diaminoalkylated amino acid building block via tandem double reductive alkylation

occurring, linear cationic antimicrobial peptide. In general, the antibacterial activity of cationic peptides can be modulated through alteration of the peptide's hydrophobicity and/or net charge. Therefore, incorporation of **5** instead of lysine residue in indolicidin will increase its net positive charge and disrupt the balance between the charge and hydrophobicity without altering the number of peptide bonds.

Both indolicidin and its analogue **Indo-5** were successfully synthesized using Fmoc-protected amino acids and Rink amide MBHA resin following standard Fmoc-SPPS protocols on an automated peptide synthesizer. Upon cleavage of the peptides from the solid support and purification by HPLC, 98 % pure peptides were obtained. The antimicrobial activities of both indolicidin and its analogue **Indo-5** were tested against multidrug-resistant strains of *Staphylococcus aureus* [ATCC 33591] and *Escherichia coli* [ATCC 29181], and the results are summarized in Table 2. Interestingly, the synthetic analogue **Indo-5**, bearing an additional positive charge, showed somewhat improved activity against both bacterial strains (MIC 32 μg/mL) in comparison with the natural product indolicidin (MIC 64 μg/mL). These results are in agreement with previous reports in which an increase in net positive charge translates into enhanced indolicidin's antimicrobial activity (Nan et al. 2009a, b). However, the obtained MIC data showed that a decrease in overall hydrophobicity, as indicated by the HPLC retention times (Table 2), did not correlate with their antibacterial activities (Lee et al. 2004; Chen et al. 2007), with less hydrophobic **Indo-5** being more active. Not surprisingly, indolicidin and **Indo-5** exhibited comparable hemolytic activities, confirming the dependence of its hemolytic activity with specific tryptophans and the arginines in the sequence (Ando et al. 2010).

The structural features of indolicidin and **Indo-5** were monitored by CD spectroscopy, Fig. 2. The CD spectra were recorded in water as well as in less polar solvent systems such as 50 and 100 % TFE. Typically, TFE is used as a membrane mimicking solvent, and it is known to induce formation of the stable conformations in peptides which are otherwise unstructured in aqueous solutions (Sonnichsen et al. 1992; Roccatano et al. 2002; Otvos 1997).

The CD spectrum of indolicidin measured in water was similar to those previously reported, displaying a single negative band at 201 nm, characteristic of an unordered conformation (Falla et al. 1996; Ando et al. 2010). However, the proposed secondary structure of indolicidin has been somewhat controversial, and poly-L-Pro II helix and β-turn structures were reported as well (Hsu et al. 2005; Ladokhin et al. 1999; Andrushchenko et al. 2006).

An increase in the TFE concentration resulted in a slight shift of the minimum from 201 to 198 nm suggesting no major conformational change. In the case of the indolicidin analogue **Indo-5**, the CD spectrum recorded in water showed an intense negative band at 201 nm along with a weak positive band at 227 nm, reminiscent of a poly-L-Pro

Table 2 Retention time, charge, antimicrobial and hemolytic activity of indolicidin (**Indo**) and its analogue (**Indo-5**)

Peptides	t_R (min)[a]	Net charge	MIC (μg/mL)[b]		% Hemolysis[e]
			S. aureus[c]	*E. coli*[d]	
Indo	19.05	+4	64	64	20
Indo-5	18.47	+5	32	32	18

[a] Analytical RP-HPLC conditions described in general methods

[b] See "Materials and methods" section

[c] ATCC 33591

[d] ATCC 29181

[e] Determined at MIC concentration of peptides and expressed in percent relative to total hemolysis caused by Triton-X-100

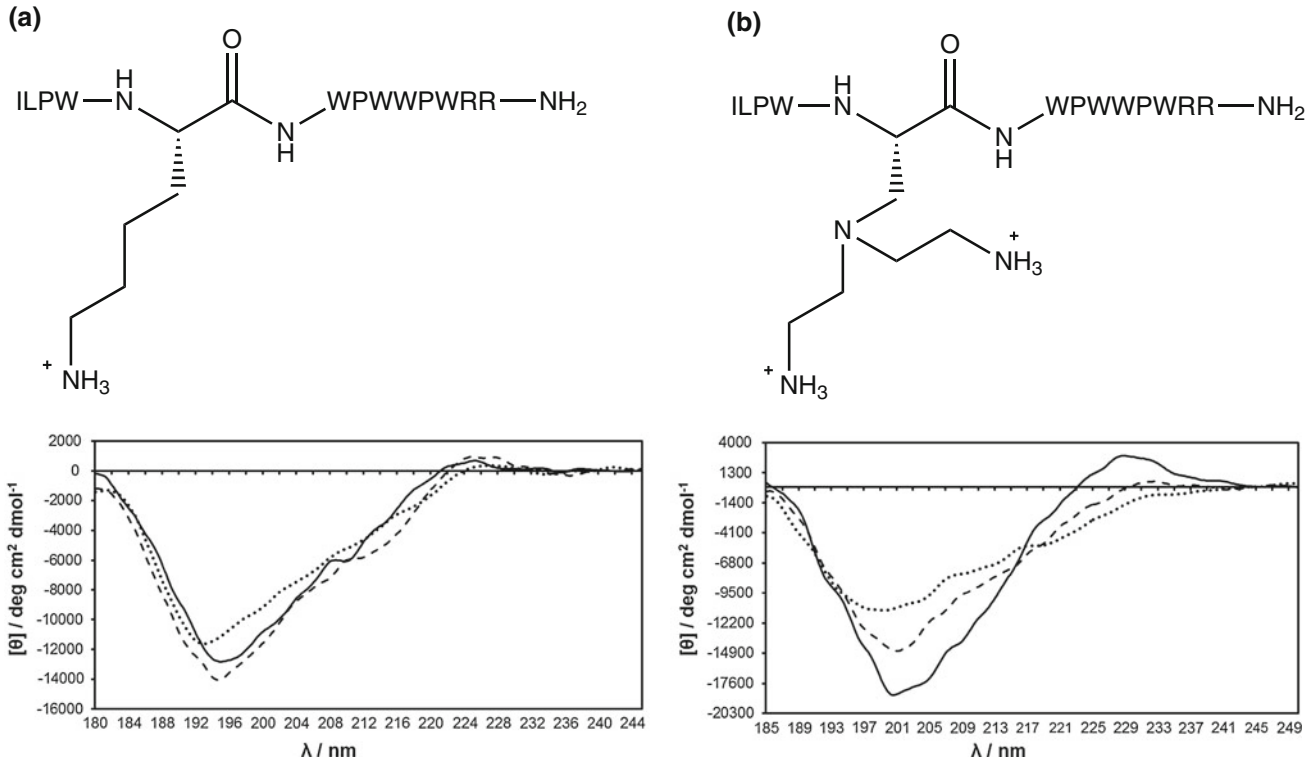


Fig. 2 Structures and CD spectra of **a** indolicidin, and **b** synthetic analogue **Indo-5** in water (*solid line*), 50 % TFE (*dashed line*), and 100 % TFE (*dotted line*)

II helix structure. However, the observed decrease in the intensity of the CD spectrum of **Indo-5** in 50 and 100 % TFE suggests appearance of a more ordered secondary structure (Otvos 1997). Obtained results suggest that the substitution of the lysine residue with our branched basic amino acids, such as **5**, can induce changes in the peptide's secondary structure.

Conclusion

We have devised a simple and efficient one-pot procedure that enables rapid access to orthogonally protected *N*,*N*′-diaminoalkylated amino acid building blocks suitable for standard Boc and Fmoc-solid-phase peptide synthesis. The synthetic strategy includes double reductive alkylation of commercially available N^{α}-protected diamino acids with *N*-protected amino aldehydes in the presence of sodium cyanoborohydride. The mild reaction conditions required for this reaction proceeds without giving rise to side-products. The advantages of this approach include versatile starting materials and access to symmetrically and unsymmetrically branched basic amino acid building blocks. In addition, the possibility of further modification of these derivatives in solution or on solid support made them well suited for potential combinatorial chemistry

diversity. Successful synthesis of an indolicidin analogue in which Lys residue was substituted with **5** demonstrated applicability of the prepared branched amino acid building blocks in standard SPPS. This substitution resulted in an indolicidin analogue with increased net positive charge, more ordered secondary structure, improved antibacterial activity, and with hemolytic activity comparable to the parent natural product.

Taking into consideration the increasing interest for peptides with unusual structural features due to their improved biological properties, the described synthesis of *N*,*N*′-diaminoalkylated basic amino acids is of particular practical value.

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References

Abdel-Magid AF, Harris BD, Maryanoff CA (1994) A reductive amination/lactamization procedure using borohydride reagents. Synlett 1:81–83

Ando S, Mitsuyasu K, Soeda Y, Hidaka M, Ito Y, Matsubara K, Shindo M, Uchida Y, Aoyagi H (2010) Structure-activity relationship of indolicidin, a Trp-rich antibacterial peptide. J Pept Sci 16(4):171–177. doi:10.1002/psc.1217

Andrushchenko VV, Vogel HJ, Prenner EJ (2006) Solvent-dependent structure of two tryptophan-rich antimicrobial peptides and their analogs studied by FTIR and CD spectroscopy. Biochim Biophys Acta 1758(10):1596–1608. doi:10.1016/j.bbamem.2006.07.013

Ariza X, Urpí F, Vilarrasa J (1999) A practical procedure for the preparation of carbamates from azides. Tetrahedron Lett 40(42): 7515–7517. doi:10.1016/s0040-4039(99)01449-5

Bartholoma M, Valliant J, Maresca KP, Babich J, Zubieta J (2009) Single amino acid chelates (SAAC): a strategy for the design of technetium and rhenium radiopharmaceuticals. Chem Commun 5:493–512

Bhushan R, Brückner H (2004) Marfey's reagent for chiral amino acid analysis: a review. Amino Acids 27(3):231–247. doi:10.1007/s00726-004-0118-0

Boeijen A, Liskamp RMJ (1999) Solid-phase synthesis of oligourea peptidomimetics. Eur J Org Chem 9:2127–2135. doi:10.1002/(sici)1099-0690(199909)1999:9<2127:aid-ejoc2127>3.0.co;2-t

Borch RF, Bernstein MD, Durst HD (1971) Cyanohydridoborate anion as a selective reducing agent. J Am Chem Soc 93(12):2897–2904. doi:10.1021/ja00741a013

Boyer JH (1951) Addition of hydrazoic acid to conjugated systems1. J Am Chem Soc 73(11):5248–5252. doi:10.1021/ja01155a073

Bräse S, Gil C, Knepper K, Zimmermann V (2005) Organic azides: an exploding diversity of a unique class of compounds. Angew Chem Int Ed 44(33):5188–5240. doi:10.1002/anie.200400657

Chang PV, Prescher JA, Sletten EM, Baskin JM, Miller IA, Agard NJ, Lo A, Bertozzi CR (2010) Copper-free click chemistry in living animals. Proc Nat Acad Sci. doi:10.1073/pnas.0911116107

Chen Y, Guarnieri MT, Vasil AI, Vasil ML, Mant CT, Hodges RS (2007) Role of peptide hydrophobicity in the mechanism of action of α-helical antimicrobial peptides. Antimicrob Agents Chemother 51(4):1398–1406. doi:10.1128/aac.00925-06

CLSI (2011) Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved Standard M7-A8. Clinical and Laboratory Standards Institute, Wayne, PA

Collum DB, Chen S-C, Ganem B (1978) A new synthesis of amides and macrocyclic lactams. J Org Chem 43(22):4393–4394. doi:10.1021/jo00416a040

Cudic P, Stawikowski M (2007) Pseudopeptide synthesis via Fmoc solid-phase synthetic methodology. Mini-Rev Org Chem 4:268–280

David SA (2001) Towards a rational development of anti-endotoxin agents: novel approaches to sequestration of bacterial endotoxins with small molecules. J Mol Recognit 14(6):370–387. doi:10.1002/jmr.549

Davies AJ, Donald ASR, Marks RE (1967) The acid-catalysed decomposition of some [small beta]-azido-carbonyl compounds. J Chem Soc C Org:2109–2112

de Jong R, Rijkers DTS, Liskamp RMJ (2002) Automated solid-phase synthesis and structural investigation of β-peptidosulfonamides and β-peptidosulfonamide/β-peptide hybrids: β-peptidosulfonamide and β-peptide foldamers are two of a different kind. Helv Chim Acta 85(12):4230–4243. doi:10.1002/hlca.200290008

Derossi D, Joliot AH, Chassaing G, Prochiantz A (1994) The third helix of the Antennapedia homeodomain translocates through biological membranes. J Biol Chem 269(14):10444–10450

Diness F, Beyer J, Meldal M (2004) Synthesis of 3-Boc-(1,3)-oxazinane-protected amino aldehydes from amino acids and their conversion into urea precursors. novel building blocks for combinatorial synthesis. QSAR Comb Sci 23(2–3):130–144. doi:10.1002/qsar.200320012

Falla TJ, Karunaratne DN, Hancock REW (1996) Mode of action of the antimicrobial peptide indolicidin. J Biol Chem 271(32): 19298–19303. doi:10.1074/jbc.271.32.19298

Fields GB, Prockop DJ (1996) Perspectives on the synthesis and application of triple-helical, collagen-model peptides. Pept Sci 40(4):345–357. doi:10.1002/(sici)1097-0282(1996)40:4<345:aid-bip1>3.0.co;2-w

Fields CG, Mickelson DJ, Drake SL, McCarthy JB, Fields GB (1993) Melanoma cell adhesion and spreading activities of a synthetic 124-residue triple-helical "mini-collagen". J Biol Chem 268(19):14153–14160

Findlay B, Zhanel GG, Schweizer F (2010) Cationic amphiphiles, a new generation of antimicrobials inspired by the natural antimicrobial peptide scaffold. Antimicrob Agents Chemother 54(10):4049–4058. doi:10.1128/aac.00530-10

Fowler SA, Blackwell HE (2009) Structure-function relationships in peptoids: recent advances toward deciphering the structural requirements for biological function. Org Biomol Chem 7(8): 1508–1524

Futaki S, Suzuki T, Ohashi W, Yagami T, Tanaka S, Ueda K, Sugiura Y (2001) Arginine-rich peptides. J Biol Chem 276(8):5836–5840. doi:10.1074/jbc.M007540200

Gante J, Krug M, Lauterbach G, Weitzel R, Hiller W (1995) Synthesis and properties of the first all-aza analogue of a biologically active peptide. J Pept Sci 1(3):201–206. doi:10.1002/psc.310010307

Gentilucci L, De Marco R, Cerisoli L (2010) Chemical modifications designed to improve peptide stability: incorporation of non-natural amino acids, pseudo-peptide bonds, and cyclization. Curr Pharm Des 16(28):3185–3203

Grab B, Miles AJ, Furcht LT, Fields GB (1996) Promotion of fibroblast adhesion by triple-helical peptide models of type i

collagen-derived sequences. J Biol Chem 271(21):12234–12240. doi:10.1074/jbc.271.21.12234
Green M, Loewenstein PM (1988) Autonomous functional domains of chemically synthesized human immunodeficiency virus tat trans-activator protein. Cell 55(6):1179–1188. doi:10.1016/0092-8674(88)90262-0
Hahn K, Klis W, Stewart J (1990) Design and synthesis of a peptide having chymotrypsin-like esterase activity. Science 248(4962):1544–1547. doi:10.1126/science.2360048
Hsu C-H, Chen C, Jou M-L, Lee AY-L, Lin Y-C, Yu Y-P, Huang W-T, Wu S-H (2005) Structural and DNA-binding studies on the bovine antimicrobial peptide, indolicidin: evidence for multiple conformations involved in binding to membranes and DNA. Nucleic Acids Res 33(13):4053–4064. doi:10.1093/nar/gki725
Jenssen H, Hamill P, Hancock REW (2006) Peptide Antimicrobial Agents. Clin Microbiol Rev 19(3):491–511. doi:10.1128/cmr.00056-05
Karskela T, Virta P, Lönnberg H (2006) Synthesis of Bicyclic Peptides. Curr Org Synth 3(3):283–311. doi:10.2174/157017906777934917
Katsara M, Tselios T, Deraos S, Deraos G, Matsoukas M-T, Lazoura E, Matsoukas J, Apostolopoulos V (2006) Round and round we go: cyclic peptides in disease. Curr Med Chem 13(19):2221–2232
Kolb HC, Finn MG, Sharpless KB (2001) Click chemistry: diverse chemical function from a few good reactions. Angew Chem Int Ed 40(11):2004–2021. doi:10.1002/1521-3773(20010601)40:11<2004:aid-anie2004>3.0.co;2-5
Ladokhin AS, Selsted ME, White SH (1999) CD spectra of indolicidin antimicrobial peptides suggest turns, not polyproline helix. Biochemistry 38(38):12313–12319. doi:10.1021/bi9907936
Lee RT, Lee YC (1980) Preparation and some biochemical properties of neoglycoproteins produced by reductive amination of thioglycosides containing an.omega.-aldehydoaglycon. Biochemistry 19(1):156–163. doi:10.1021/bi00542a024
Lee DL, Powers JPS, Pflegerl K, Vasil ML, Hancock REW, Hodges RS (2004) Effects of single D-amino acid substitutions on disruption of β-sheet structure and hydrophobicity in cyclic 14-residue antimicrobial peptide analogs related to gramicidin S. J Pept Res 63(2):69–84. doi:10.1046/j.1399-3011.2003.00106.x
Levadala MK, Banerjee SR, Maresca KP, Babich JW, Zubieta J (2004) Direct reductive alkylation of amino acids: synthesis of bifunctional chelates for nuclear imaging. Synthesis 2004(11):1759–1766. doi:10.1055/s-2004-829120
Liskamp RMJ, Rijkers DTS, Kruijtzer JAW, Kemmink J (2011) Peptides and proteins as a continuing exciting source of inspiration for peptidomimetics. ChemBioChem 12(11):1626–1653. doi:10.1002/cbic.201000717
Lundquist JT, Pelletier JC (2001) Improved solid-phase peptide synthesis method utilizing α-azide-protected amino acids. Org Lett 3(5):781–783. doi:10.1021/ol0155485
Lundquist JT, Pelletier JC (2002) A new tri-orthogonal strategy for peptide cyclization. Org Lett 4(19):3219–3221. doi:10.1021/ol026416u
Lutz J-F (2008) Copper-free azide–alkyne cycloadditions: new insights and perspectives. Angew Chem Int Ed 47(12):2182–2184. doi:10.1002/anie.200705365
Mutter M, Tuchscherer GG, Miller C, Altmann KH, Carey RI, Wyss DF, Labhardt AM, Rivier JE (1992) Template-assembled synthetic proteins with four-helix-bundle topology. Total chemical synthesis and conformational studies. J Am Chem Soc 114(4):1463–1470. doi:10.1021/ja00030a049
Nan YH, Bang J-K, Shin SY (2009a) Design of novel indolicidin-derived antimicrobial peptides with enhanced cell specificity and potent anti-inflammatory activity. Peptides 30(5):832–838. doi:10.1016/j.peptides.2009.01.015
Nan YH, Park KH, Park Y, Jeon YJ, Kim Y, Park I-S, Hahm K-S, Shin SY (2009b) Investigating the effects of positive charge and hydrophobicity on the cell selectivity, mechanism of action and anti-inflammatory activity of a Trp-rich antimicrobial peptide indolicidin. FEMS Microbiol Lett 292(1):134–140. doi:10.1111/j.1574-6968.2008.01484.x
Ning X, Guo J, Wolfert MA, Boons G-J (2008) Visualizing metabolically labeled glycoconjugates of living cells by copper-free and fast huisgen cycloadditions. Angew Chem Int Ed 47(12):2253–2255. doi:10.1002/anie.200705456
Novabiochem Catalog (2010/2011) Peptide Synthesis. Darmstadt, Germany
Oehlke J, Scheller A, Wiesner B, Krause E, Beyermann M, Klauschenz E, Melzig M, Bienert M (1998) Cellular uptake of an α-helical amphipathic model peptide with the potential to deliver polar compounds into the cell interior non-endocytically. Biochim Biophys Acta 1414(1–2):127–139. doi:10.1016/s0005-2736(98)00161-8
Otvos L (1997) Use of circular dichroism to determine secondary structure of neuropeptides neuropeptide protocols. In: Irvine GB, Williams CH (eds) Methods in molecular biology. Humana Press, vol 73, pp 153–161. doi:10.1385/0-89603-399-6:153
Patel L, Zaro J, Shen W-C (2007) Cell penetrating peptides: intracellular pathways and pharmaceutical perspectives. Pharm Res 24(11):1977–1992. doi:10.1007/s11095-007-9303-7
Reggelin M, Junker B, Heinrich T, Slavik S, Bühle P (2006) Asymmetric synthesis of highly substituted azapolycyclic compounds via 2-alkenyl sulfoximines: potential scaffolds for peptide mimetics. J Am Chem Soc 128(12):4023–4034. doi:10.1021/ja057012a
Roccatano D, Colombo G, Fioroni M, Mark AE (2002) Mechanism by which 2,2,2-trifluoroethanol/water mixtures stabilize secondary-structure formation in peptides: a molecular dynamics study. Proc Nat Acad Sci 99(19):12179–12184. doi:10.1073/pnas.182199699
Rostovtsev VV, Green LG, Fokin VV, Sharpless KB (2002) A stepwise huisgen cycloaddition process: copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes. Angew Chem Int Ed 41(14):2596–2599. doi:10.1002/1521-3773(20020715)41:14<2596:aid-anie2596>3.0.co;2-4
Sadler K, Tam JP (2002) Peptide dendrimers: applications and synthesis. Rev Mol Biotechnol 90(3–4):195–229. doi:10.1016/s1389-0352(01)00061-7
Sebestik J, Niederhafner P, Jezek J (2011) Peptide and glycopeptide dendrimers and analogous dendrimeric structures and their biomedical applications. Amino Acids 40(2):301–370. doi:10.1007/s00726-010-0707-z
Selsted ME, Novotny MJ, Morris WL, Tang YQ, Smith W, Cullor JS (1992) Indolicidin, a novel bactericidal tridecapeptide amide from neutrophils. J Biol Chem 267(7):4292–4295
Solá RJ, Griebenow K (2010) Glycosylation of therapeutic proteins: an effective strategy to optimize efficacy. BioDrugs 24(1):9–21. doi:10.2165/11530550-000000000-000000000
Sonnichsen FD, Van Eyk JE, Hodges RS, Sykes BD (1992) Effect of trifluoroethanol on protein secondary structure: an NMR and CD study using a synthetic actin peptide. Biochemistry 31(37):8790–8798. doi:10.1021/bi00152a015
Stevenson CL (2009) Advances in peptide pharmaceuticals. Curr Pharm Biotechnol 10(1):122–137
Tolle JC, Staples MA, Blout ER (1982) Synthesis of a new type of cyclic peptide: a bicyclic nonapeptide. J Am Chem Soc 104(24):6883–6884. doi:10.1021/ja00388a114
Toniolo C (2004) Peptides incorporating secondary structure inducers and mimetics. In: Goodman M (ed) Synthesis of peptides and peptidomimetics, vol E., 22cHouben-Weyl, Stuttgart, pp 693–835
Tornøe CW, Christensen C, Meldal M (2002) Peptidotriazoles on solid phase: [1,2,3]-triazoles by regiospecific copper(I)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides. J Org Chem 67(9):3057–3064. doi:10.1021/jo011148j

Tsubery H, Ofek I, Cohen S, Fridkin M (2000) Structure-Function Studies of Polymyxin B Nonapeptide: implications to Sensitization of Gram-Negative Bacteria. J Med Chem 43(16):3085–3092. doi:10.1021/jm0000057

Wender PA, Mitchell DJ, Pattabiraman K, Pelkey ET, Steinman L, Rothbard JB (2000) The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: peptoid molecular transporters. Proc Nat Acad Sci 97(24):13003–13008. doi:10.1073/pnas.97.24.13003

White PD, Chan WC (2003) Basic principles. In: White PD, Chan WC (eds) Fmoc solid phase peptide synthesis. a practical approach. practical approach series, vol 222, Oxford University Press, Eynsham, pp 9–40

White CJ, Yudin AK (2011) Contemporary strategies for peptide macrocyclization. Nat Chem 3(7):509–524

Yoo B, Shin SBY, Huang ML, Kirshenbaum K (2010) Peptoid macrocycles: making the rounds with peptidomimetic oligomers. chemistry—a. Chem Eur J 16(19):5528–5537. doi:10.1002/chem.200903549

Yu C, Taylor JW (1996) A new strategy applied to the synthesis of an [alpha]-helical bicyclic peptide constrained by two overlapping i, i + 7 side-chain bridges of novel design. Tetrahedron Lett 37(11):1731–1734. doi:10.1016/0040-4039(96)00121-9

Zega A (2005) Azapeptides as pharmacological agents. Curr Med Chem 12(5):589–597

Zhang W, Taylor JW (1996) Efficient solid-phase synthesis of peptides with tripodal side-chain bridges and optimization of the solvent conditions for solid-phase cyclizations. Tetrahedron Lett 37(13):2173–2176. doi:10.1016/0040-4039(96)00220-1

Zuckermann RN, Kerr JM, Kent SBH, Moos WH (1992) Efficient method for the preparation of peptoids [oligo(N-substituted glycines)] by submonomer solid-phase synthesis. J Am Chem Soc 114(26):10646–10647. doi:10.1021/ja00052a076